Behavioral sensitization to amphetamine results from an uncoupling between noradrenergic and serotonergic neurons

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In rodents, drugs of abuse induce locomotor hyperactivity, and repeating injections enhances this response. This effect, called behavioral sensitization, persists many months after the last administration, thus mimicking long-term sensitivity to drugs observed in human addicts. We show here that, in naïve animals, noradrenergic and serotonergic systems, besides their behavioral activating effects, inhibit each other by means of the stimulation of α1b-adrenergic and 5-HT_{2A} receptors and that this mutual inhibition vanishes with repeated injections of *d*-amphetamine; this uncoupling may be responsible for behavioral sensitization and for an increased reactivity of dopaminergic neurons. First, after repeated *d*-amphetamine injections, a *d*-amphetamine challenge induces a dramatic increase in cortical extracellular norepinephrine (NE) levels. This increased cortical NE release still occurs after 1 month of withdrawal but is diminished or blocked if sensitization is performed in the presence of prazosin, SR46349B, or both α1-adrenergic and 5-HT_{2A} receptor antagonists, respectively. A strong correlation between increases in cortical extracellular NE levels and the expression of behavioral sensitization was found. Second, repeated *d*-amphetamine injections induce an increased reactivity of serotonergic neurons measured by cortical extracellular serotonin (5-HT) levels after the administration of a 5-HT releaser, *p*-chloroamphetamine. Third, knockout mice for α1b-adrenergic (α1b-AR KO) or 5-HT_{2A} (5-HT_{2A}-R KO) receptor, respectively, exhibit a behavioral and biochemical hyperreactivity to the acute injection of *p*-chloroamphetamine (α1b-AR KO; 5-HT levels) and *d*-amphetamine (5-HT_{2A}-R KO; NE levels). Uncoupling between noradrenergic and serotonergic neurons may occur not only in addiction but also during chronic stressful situations, thus facilitating the onset of mental illness.

d-amphetamine | microdialysis | norepinephrine | serotonin | behavioral sensitization

Psychostimulants and opiates, two major groups of drugs of abuse, produce locomotor stimulant effects that become enhanced with repeated intermittent injections. This enhanced behavioral response, named behavioral sensitization, is enduring and can last up to 1 year after drug exposure (1). Studies of the neurobiological basis of behavioral sensitization have focused, despite conflicting data (2–6), on the midbrain dopamine (DA) system because of evidence suggesting that this system mediates locomotor stimulation as well as the ability of drugs to elicit craving and to lead to abuse (7). Indeed, it was established that most drugs abused by humans increase DA release in the nucleus accumbens, a structure innervated by midbrain DA neurons (8). Moreover, animals readily self-administer agents that increase DA transmission, such as amphetamine and cocaine (9). Furthermore, it has been proposed that the rewarding properties of opiates, such as morphine or heroin, are produced by the disinhibition of midbrain DA cells firing via the stimulation of μ-opiate receptors located on GABAergic midbrain interneurons that negatively regulate DA cells firing (10). Recently, however, we have shown that psychostimulant/opiate-induced locomotor stimulation and behavioral sensitization are entirely dependent on the stimulation of two nondopaminergic monoaminergic receptors, α1b-adrenergic and 5-HT_{2A} (11). Knockout mice for the α1b-adrenergic receptor (α1b-AR KO) (12) and the antagonists of α1-adrenergic and 5-HT_{2A} receptors (prazosin and SR46349B, respectively) were used to define α1b-adrenergic and 5-HT_{2A} components in drug-induced locomotor activity. We have shown that prazosin blocks most of the morphine-evoked locomotor response in WT mice and that, as expected, morphine-evoked locomotor response in α1b-AR KO mice was not affected by prazosin (11). Surprisingly, morphine-evoked locomotor response was 3-fold higher in α1b-AR KO mice than in WT mice when both species were treated with prazosin (11). Because SR46349B entirely inhibited morphine-induced locomotor response in α1b-AR KO mice, it was suggested that 5-HT_{2A} receptors could compensate for the genetic deletion of α1b-adrenergic receptors (11). However, when both species were repeatedly treated with morphine, morphine-evoked locomotor response in presence of prazosin increased in WT mice and became similar to that observed in α1b-AR KO mice (11). This finding suggests that prazosin limits the 5-HT_{2A} component of morphine-evoked locomotor activity in naïve WT mice and that this limitation disappears when animals are sensitized. A possibility could be that, in addition to their behavioral activating effects, noradrenergic and serotonergic systems are coupled (i.e., limit or stimulate each other) in naïve animals and become independent after repeated injections of psychostimulants or opiates, explaining accordingly the development of behavioral sensitization.

To test the first hypothesis, i.e., whether 5-HT_{2A} receptors compensate for the genetic deletion of α1b-adrenergic receptors and also whether α1b-adrenergic receptors compensate in 5-HT_{2A} receptor knockout mice (5-HT_{2A}-R KO) (13), densities of α1b-adrenergic and 5-HT_{2A} binding sites were measured by autoradiography in 5-HT_{2A}-R KO and α1b-AR KO mice and compared with those of WT mice. Binding sites were studied in the prefrontal cortex (PFC) because of the role of cortical α1b-adrenergic receptors in stimulant-induced locomotor activity (14, 15) and because both receptors are colocalized in layers III–V in this structure (14, 16). The absence of obvious interactions between α1b-adrenergic and 5-HT_{2A} receptors led us to study, as mentioned above, the possibility of a modification of a mutual relationship between noradrenergic and serotonergic neurons after repeated treatments

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Abbreviations: DA, dopamine; NE, norepinephrine; 5-HT, serotonin; PCA, p-chloroamphetamine; VTA, ventral tegmental area; PFC, prefrontal cortex; α1b-AR KO, knockout for the α1b-adrenergic receptor; 5-HT_{2A}-R KO, knockout for the 5-HT_{2A} receptor.

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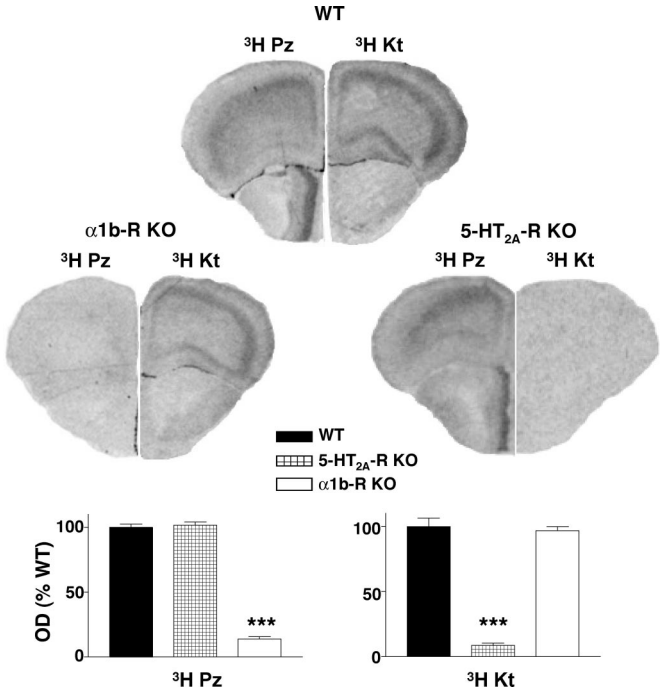

Fig. 1. Autoradiography of α1b-adrenergic and 5-HT_{2A} binding sites in α1b-AR KO and 5-HT_{2A}-R KO mice and a comparison with WT mice. The higher amplitude of the 5-HT_{2A} locomotor component observed in α1b-AR KO mice (11), when compared with WT, is not due to an increased density in 5-HT_{2A} receptor binding sites. Similarly, the density of cortical α1b-adrenergic binding sites is not modified in 5-HT_{2A}-R KO mice. 3H Kt, tritiated ketanserin; 3H Pz, tritiated prazosin. ***, $P <$ 0.001 when compared with corresponding controls.

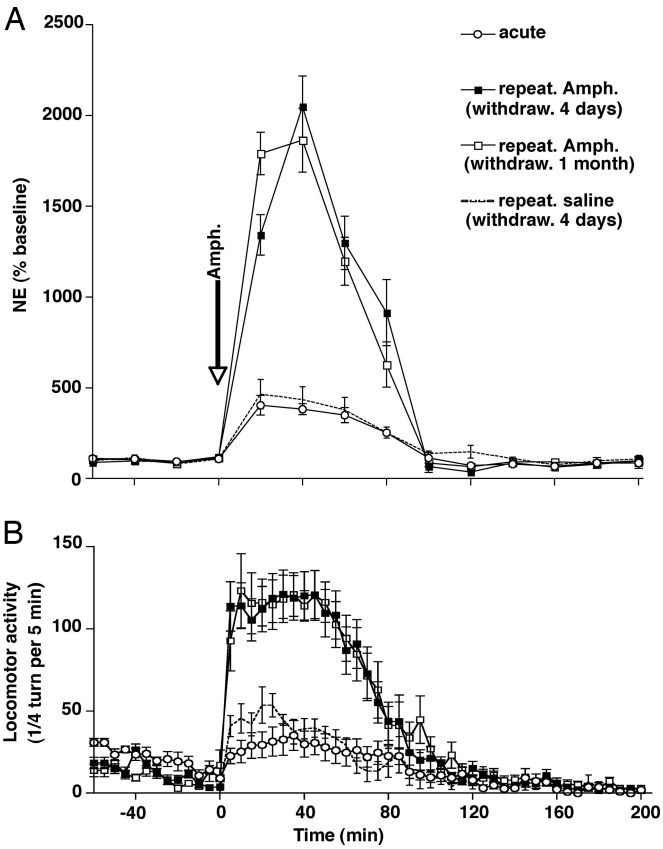

Fig. 2. Repeated treatment with *d*-amphetamine induces both a behavioral sensitization and a potentiation in the increase of cortical extracellular NE levels that lasts for at least 1 month. Cortical extracellular NE levels are expressed as a percentage of the respective mean basal value (acute, 0.64 ± 0.1 pg of NE every 20 min; repeated 4-days withdrawal, 0.55 ± 0.09 pg every 20 min; 1-month withdrawal, 0.56 ± 0.1 pg every 20 min). Effects of acute saline injection, which did not significantly modify basal cortical extracellular NE levels, are not shown for the sake of clarity. Each group contained at least seven animals to determine locomotor activity and five animals in microdialysis experiments.

with psychostimulants or opiates. We present here data obtained with repeated *d*-amphetamine treatments.

First, mice locomotor responses and prefrontocortical extracellular norepinephrine (NE) levels were determined in different conditions, i.e., after acute and repeated treatments with *d*-amphetamine, after 4 days or 1 month of withdrawal and when repeated *d*-amphetamine treatments were done in presence of prazosin, SR46349B, or a mixture of both antagonists. Then, extracellular prefrontocortical serotonin (5-HT) levels were determined in naïve animals and in those having received repeated *d*-amphetamine treatments. However, because *d*-amphetamine did not modify cortical 5-HT extracellular levels in our experimental conditions, reactivity of serotonergic neurons was estimated after the injection of a 5-HT releaser, p-chloroamphetamine (PCA), a compound analogous to ecstasy and known to induce locomotor hyperactivity and behavioral sensitization in mice (17).

Finally, locomotor and biochemical responses to *d*-amphetamine or PCA were measured in 5-HT_{2A}-R KO or α1b-AR KO mice, respectively.

Results

Absence of Direct Interactions Between α1b-Adrenergic and 5-HT_{2A} Receptors. Fig. 1 shows that, whereas binding of tritiated prazosin is almost completely abolished ($P < 0.001$) in α1b-AR KO mice, there is no change in tritiated ketanserin binding when compared with WT mice. Similarly, in 5-HT_{2A}-R KO mice, despite the important decrease in tritiated ketanserin binding ($P < 0.001$), no change occurs in tritiated prazosin binding when compared with WT mice. This result clearly indicates that α1b-adrenergic and 5-HT_{2A} receptors do not compensate for each other and suggests that the dependency between both systems observed in naïve animals is due to interactions that are presynaptic to α1b-adrenergic and 5-HT_{2A} receptors, i.e., between noradrenergic and serotonergic neurons.

Effects of Acute and Repeated *d*-Amphetamine on *d*-Amphetamine-Induced Locomotor Responses and Cortical Extracellular NE Levels. Fig. 2 illustrates that, as expected, acute injection of *d*-amphetamine increases both locomotor activity [$F(1,273) = 166.6$; $P < 0.0001$] and extracellular NE levels in the PFC [+167%; $F(1,5) = 172.7$; $P < 0.0001$, when compared with basal values] (18). After a 4-day withdrawal period, animals that received repeated injections of *d*-amphetamine not only exhibited an important behavioral sensitization [+241%; $F(1,20) = 315.1$; $P < 0.0001$, when compared with acute] but also a dramatic and similar potentiation in extracellular NE levels [+259%; $F(1,5) = 116.9$; $P < 0.0001$, when compared with acute]. Interestingly, after a 1-month withdrawal period, locomotor responses, as well as extracellular NE levels, exhibited a potentiation similar to that observed after 4 days [$F(1,252) = 0.2620$, $P = 0.6092$; and $F(1,54) = 0.0664$, $P = 0.7976$, respectively). Moreover, the time course of the locomotor response and extracellular NE level appeared strikingly similar in the different experimental conditions (Fig. 2 *A* and *B*). Repeated saline was not different from acute for NE levels [$F(1,37) = 1.157$, $P = 0.29$] and was 20% higher than acute for locomotor response [$F(1,269) = 6.107$, $P = 0.014$].

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Effects of Pretreatment Before Repeated *d*-Amphetamine with Prazosin, SR46349B, or a Mixture of both, on *d*-Amphetamine-Induced Locomotor Response and Cortical Extracellular NE Levels. To further confirm this coincidence of behavioral response and shift in cortical extracellular NE levels, animals were pretreated with 1 mg/kg prazosin, 1 mg/kg SR46349B or with a mixture of 1 mg/kg prazosin and 1 mg/kg SR46349B during the sensitization period with *d*-amphetamine. These three conditions either completely block behavioral sensitization (mixture pretreatment) or diminish it by 75% or 85% (prazosin and SR46349B pretreatment, respectively) (11). In this current study, the effect of these pretreatments on behavioral sensitization was confirmed (Fig. 3). When sensitization was performed in the presence of both antagonists, *d*-amphetamine induced on the test day a locomotor activity slightly lower than acute [−15%; $F(1,43) = 15.1$, $P = 0.0003$], and extracellular NE levels were slightly lower than in animals treated acutely [−12%; $F(1,43) = 15.14$, $P < 0.001$]. When pretreated with *d*-amphetamine and prazosin, *d*-amphetamine on the test day induced a locomotor response significantly higher than acute [+59%; $F(1,20) = 26.3$, $P < 0.0001$] but lower than repeated [−53%; $F(1,20) = 123.5$, $P < 0.0001$] as well as extracellular NE levels that were significantly higher than acute [+58%; $F(1,5) = 11.26$, $P < 0.005$] but lower than repeated [−54%; $F(1,5) = 105.3$, $P < 0.0001$] (Fig. 3). When pretreated with *d*-amphetamine and SR46349B, both locomotor and extracellular NE levels responses to *d*-amphetamine on the test day were not significantly different from acute [$F(1,273) = 0.0433$, $P = 0.853$; and $F(1,5) = 0.6083$, $P = 0.4397$, respectively]. Altogether, a very significant correlation ($r^2 = 0.99$) was found between *d*-amphetamine-induced cortical extracellular NE levels and locomotor response in these different conditions (Fig. 3*C*).

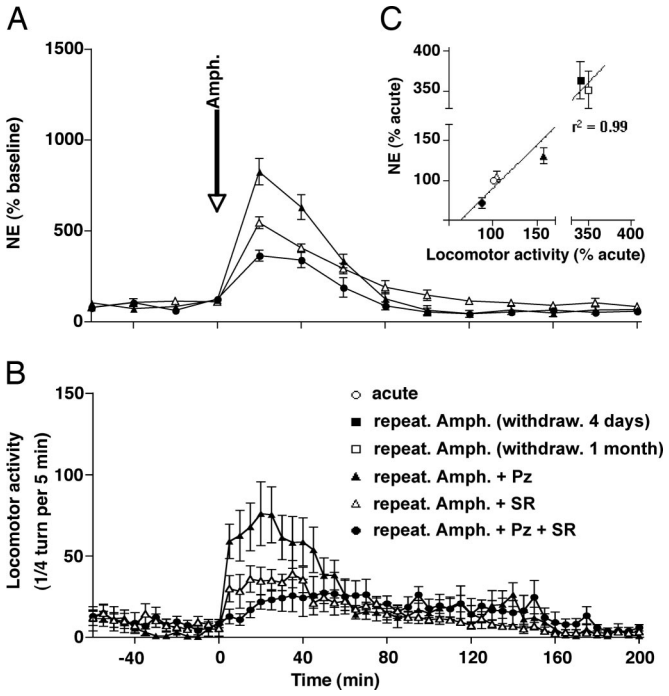

Fig. 3. Effects of pretreatment with prazosin, SR46349B, or a mixture of both on *d*-amphetamine-induced locomotor response and cortical extracellular NE levels. Cortical extracellular NE levels are expressed as a percentage of the respective mean basal value [prazosin (Pz) pretreatment, 0.54 ± 0.12 pg every 20 min; SR46349B (SR) pretreatment, 0.62 ± 0.11 pg every 20 min; prazosin plus SR46349B (Pz + SR) pretreatment, 0.49 ± 0.13 pg every 20 min). Locomotor activity after saline injection was almost equal to zero and is not shown. Effects of saline injection, which did not significantly modify basal cortical extracellular NE levels, are not shown for the sake of clarity. Each group contained at least seven animals to determine locomotor activity and five animals in microdialysis experiments. Correlation was performed on all groups by using the acute behavioral and cortical extracellular NE response totalized on 100 min after injection as the 100% basis.

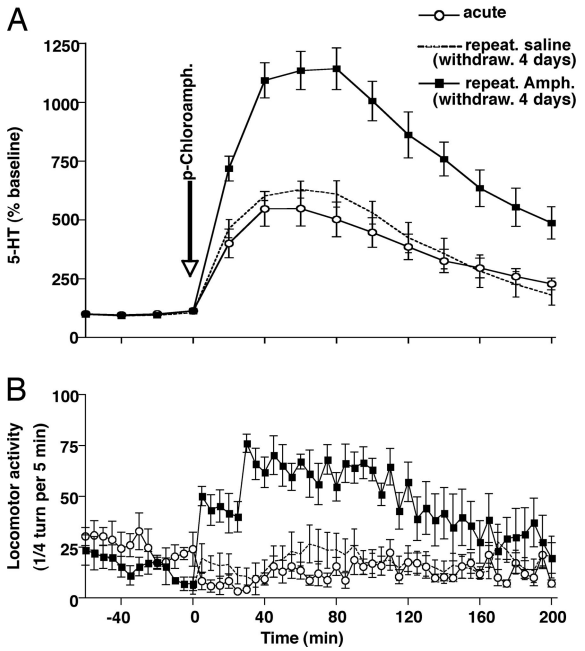

Fig. 4. Effects of PCA on locomotor and cortical extracellular 5-HT level responses in naïve mice and in those repeatedly treated with *d*-amphetamine. Cortical extracellular 5-HT levels are expressed as a percentage of the respective mean basal value (acute, 0.78 ± 0.03 pg of 5-HT every 20 min; repeated 4-day withdrawal, 0.76 ± 0.06 pg every 20 min). Effects of acute saline injection, which did not significantly modify basal cortical extracellular 5-HT levels, are not shown for the sake of clarity. Each group contained at least seven animals to determine locomotor activity and five animals in microdialysis experiments.

Effects of Repeated *d*-Amphetamine on the Increase in Cortical Extracellular 5-HT Levels Induced by PCA. As previously mentioned, in our experimental conditions 2 mg/kg *d*-amphetamine did not induce any significant change in cortical extracellular 5-HT levels in both naïve and sensitized animals (data not shown). We therefore used a moderate (7 mg/kg) (17) dose of PCA to estimate serotonergic neuron reactivity in naïve animals and in those having received a repeated treatment with *d*-amphetamine. Fig. 4 shows that an acute injection of PCA in naïve WT mice induces both a locomotor response [$F(1,231) = 66.9$, $P < 0.0001$] and a 326% increase in extracellular 5-HT levels [$F(1,5) = 104.6$, $P < 0.0001$]. In animals having received four injections of *d*-amphetamine (2 mg/kg), after a 4-day withdrawal the same dose of PCA induces a 395% increase in locomotor activity when compared with naïve animals [$F(1,20) = 469.5$, $P < 0.0001$] and a 104% increase in cortical extracellular 5-HT levels when compared with those of naïve animals [$F(1,5) = 121.7$, $P < 0.0001$]. Repeated saline was not different from acute for 5-HT levels [$F(1,607) = 3.815$, $P = 0.06$] and was 37% higher than acute for locomotor response [$F(1,225) = 7.363$, $P = 0.007$].

Effects of Acute *d*-Amphetamine and PCA on Locomotor and Cortical Extracellular NE and 5-HT Level Responses in 5-HT_{2A}-R KO and α1b-AR KO Mice, Respectively. If, as we propose, noradrenergic and serotonergic transmissions limit each other through α1b-adrenergic and 5-HT_{2A} receptors, it can be assumed that acute *d*-amphetamine-induced cortical extracellular NE levels would be higher in 5-HT_{2A}-R KO mice than in WT mice. Similarly, cortical extracellular 5-HT levels induced by PCA would be higher in α1b-AR KO

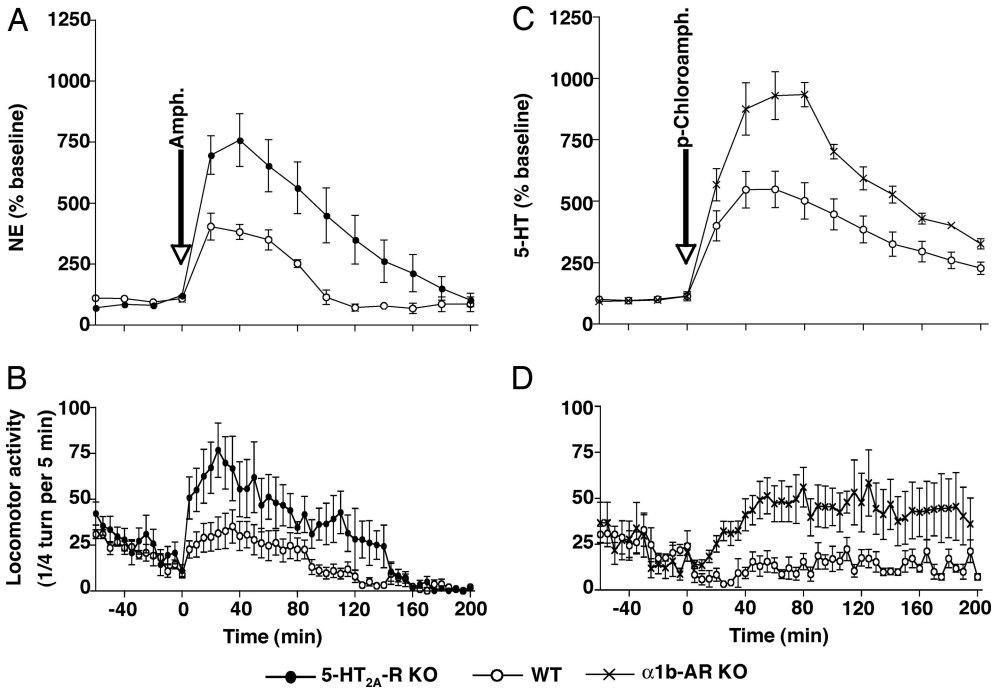

Fig. 5. Genetic deletion of α1b-adrenergic or 5-HT_{2A} receptor leads to a hyperreactivity of serotonergic or noradrenergic neurons, respectively, associated with an increased locomotor response. (*A* and *B*) *d*-amphetamine (2 mg/kg) was injected in WT and 5-HT_{2A}-R KO mice to determine locomotor activity and cortical extracellular NE levels. (*C* and *D*) PCA (7 mg/kg) was injected in WT and α1b-AR KO mice to determine locomotor activity and cortical extracellular 5-HT levels. Locomotor activity after saline injection was almost equal to zero and is not shown. Cortical extracellular NE and 5-HT levels are expressed as a percentage of the respective mean basal value (0.64 $\pm$ 0.1 pg of NE every 20 min and 0.61 $\pm$ 0.12 pg of NE every 20 min for WT and 5-HT_{2A}-R KO mice, respectively; 0.78 $\pm$ 0.03 pg of 5-HT every 20 min and 0.80 $\pm$ 0.04 pg of 5-HT every 20 min for WT and α1b-AR KO mice, respectively). Each group contained at least seven animals to determine locomotor activity and five animals in microdialysis experiments.

mice than in WT littermates. Fig. 5 *A* and *B* shows that, indeed, an acute injection of *d*-amphetamine (2 mg/kg) in 5-HT_{2A}-R KO induces both a higher locomotor response [+97%; $F(1,273) = 58.58, P < 0.0001$] and a higher increase in cortical extracellular NE levels [+101%; $F(1,49) = 31.65, P < 0.0001$] than in WT mice. In parallel, an increase in cortical extracellular 5-HT levels in α1b-AR KO mice is induced by PCA (7 mg/kg). As shown on Fig. 5 *C* and *D*, an acute injection of PCA in naïve WT mice induces, as shown previously on Fig. 4, both a locomotor response [$F(1,231) = 66.9$, $P < 0.0001$] and a 326% increase in extracellular 5-HT levels [$F(1,5) = 104.6, P < 0.0001$]. Locomotor activity and increases in cortical extracellular 5-HT levels induced by acute PCA are, however, significantly higher in α1b-AR KO mice than in WT mice [+230%, $F(1,210) = 147.4, P < 0.0001$; and +66%, $F(1,57) = 22.1$, $P < 0.0001$; for locomotor activity and cortical extracellular 5-HT levels, respectively].

Discussion

The first principal finding of this study is that the increase in extracellular NE levels in the PFC can be considered as a reliable biochemical index of *d*-amphetamine-induced locomotor activity, including its relationship with the development, the expression, and the maintenance of behavioral sensitization. Parallel with the increased *d*-amphetamine-induced locomotor response due to behavioral sensitization, responses in cortical extracellular NE levels are increased as soon as 4 days after withdrawal, and this increase stays constant at least up to 1 month later. Our results on extracellular NE levels agree with numerous data describing the critical role of cortical noradrenergic transmission in locomotor activity and its stimulatory effect on dopaminergic neurons innervating subcortical structures such as the nucleus accumbens (15, 19–21). Because of the coupling between noradrenergic and dopaminergic neurons (15, 21), our findings confirm that a relationship between behavioral sensitization and the dopaminergic subcortical system exists but suggest that the methodology used to study extracellular DA levels should be revisited. Indeed, extracellular DA levels in the nucleus accumbens after acute and repeated treatments with psychostimulants increase, stay unchanged, or even decrease with the development of behavioral sensitization (2–6). When increases were observed, they generally occurred after 2 weeks or more of withdrawal. An absence of correlation between the development of behavioral sensitization and changes in subcortical extracellular DA levels can probably be explained by stimulant-induced nonfunctional DA release in the nucleus accumbens (15, 20). This nonfunctional DA release seems related to a blunted DA reuptake activity and a clearing of DA from vesicular stores (15). Nonfunctional DA release may vary with time and hamper the determination of the functional part of the DA release during repeated treatments and early withdrawal. It may be recalled that cortical NE release induced by low doses of *d*-amphetamine was shown to be strictly dependent on impulse flow (18). This result suggests that amphetamine-induced cortical extracellular NE levels measured by microdialysis are functional, whereas, because effects of low doses of *d*-amphetamine on subcortical extracellular DA levels in the nucleus accumbens are at least partly impulse flow-independent, *d*-amphetamine would give rise to nonfunctional DA (15, 18).

The second principal finding of this study is that repeated treatments with *d*-amphetamine increase reactivity of both noradrenergic and serotonergic neurons. Because the development of this hyperreactivity can be blocked by α1b-adrenergic and 5-HT_{2A} receptor antagonist pretreatments, we propose that both types of neurons regulate each other through these two receptors in naïve animals and that this control vanishes after repeated *d*-amphetamine. The disappearance of this coupling would be the

cause of a higher reactivity of noradrenergic and serotonergic neurons and thus the expression of behavioral sensitization through an increased reactivity of dopaminergic neurons. Together with *d*-amphetamine-induced behavioral sensitization, increased reactivity of noradrenergic neurons to *d*-amphetamine can be blocked partly or completely by prazosin, SR46349B, or a mixture of both antagonists used for pretreatment. These data indicate that the stimulation of both receptors, α1b-adrenergic and 5-HT_{2A}, is implicated in the increased reactivity of noradrenergic neurons due to repeated *d*-amphetamine. Although not all of these experiments have been reproduced with PCA, it is very likely that the same effects of antagonists would occur. Indeed, we show here the inhibiting influence of each receptor on noradrenergic and serotonergic transmissions with the demonstration, in α1b-AR KO and 5-HT_{2A}-R KO mice, of a behavioral and biochemical hyperreactivity to the acute injection of the indirect agonist of the complementary neurons, (i.e., *d*-amphetamine and NE release in 5-HT_{2A}-R KO mice and PCA and 5-HT release in α1b-AR KO mice). However, the "constitutive" behavioral sensitization that knockout mice exhibit appears, at least for 5-HT_{2A}-R KO mice, to be only partial. There could be two reasons for this: first, one receptor is missing in these mutant mice, and it is likely that each receptor has its part in the behavioral activation; second, because animals were not treated repeatedly, the remaining receptor was not repeatedly stimulated, and this could hamper the development of a complete behavioral sensitization.

The dopaminergic D1 receptor is another monoaminergic receptor whose blockade was shown to inhibit the development and expression of behavioral sensitization to *d*-amphetamine (22). We pretreated animals with systemic SCH23390, a D1 antagonist, before *d*-amphetamine repeated injections and found that, after a 4-day withdrawal period, both cortical *d*-amphetamine-induced extracellular NE levels and PCA-induced extracellular 5-HT levels were identical to those observed in naïve animals (Fig. 6, which is published as supporting information on the PNAS web site). This finding confirms that these biochemical indexes covary with behavioral sensitization to *d*-amphetamine and also indicates that D1 receptor stimulation participates in noradrenergic and serotonergic neuron regulation. We also tested the biochemical consequence of the blockade by SCH23390 of the expression of behavioral sensitization to *d*-amphetamine. SCH23390 was injected, in animals previously sensitized to *d*-amphetamine, 30 min before an injection of either *d*-amphetamine or PCA. In these conditions, locomotor response to both compounds was completely blocked, and cortical *d*-amphetamine-induced extracellular NE level increases stayed 3-fold above those obtained after an acute injection, whereas PCA-induced extracellular 5-HT levels became identical to those of acutely treated animals (Fig. 7, which is published as supporting information on the PNAS web site). These results may suggest that the noradrenergic neurons are engaged upstream to D1 transmission whereas serotonergic cells would act downstream. However, besides its anti-D1 property, SCH23390 is also a potent $5HT_{2C}$ agonist (23), and this characteristic may limit the reactivity of noradrenergic and serotonergic neurons to *d*-amphetamine and PCA, respectively. Obviously, this issue needs further investigation.

Although the precise mechanism responsible for the dysregulation of noradrenergic and serotonergic neurons after repeated stimulations of α1b-adrenergic and 5-HT_{2A} receptors is not yet known, anatomical relationships between the dorsal raphe and the pontine noradrenergic nuclei suggest the existence of a functional interaction between noradrenergic and serotonergic neurons. Both neuronal groups are REM-off (24, 25), and the discharge rate of serotonergic neurons is under the excitatory control of α1-adrenergic receptors (26, 27). Conversely, raphe nuclei serotonergic cells may contact through 5-HT_{2A} receptors GABAergic interneurons that hyperpolarize noradrenergic cells in the locus coeruleus (28). Another nonexclusive possibility is that coupling between both neurotransmitter systems occurs in the PFC, where α1b-adrenergic and 5-HT_{2A} receptors are colocalized. In that case, increased cortical extracellular NE and 5-HT levels would stimulate glutamatergic pyramidal cells (29) that excite ventral tegmental area (VTA) dopaminergic neurons. However, although local injection of prazosin into the PFC blocked amphetamine-induced locomotor response (15), local bilateral injections of SR46349B into the PFC or into the VTA have indicated that only those injections done into the VTA could counteract *d*-amphetamine-induced locomotor activity (30), thus suggesting that the 5-HT_{2A} receptors implicated in the effects we observe are preferentially located in the VTA. Altogether, one can postulate that in naïve animals the activation of noradrenergic cells by external stimuli is immediately attenuated by serotonergic cells whose activation is itself triggered by noradrenergic neurons. Preliminary data obtained in the laboratory indicate that, similarly to *d*-amphetamine, repeated morphine induces the same uncoupling between noradrenergic and serotonergic neurons. Finally, we propose that this long-term uncoupling between noradrenergic and serotonergic neurons may explain the extreme sensitivity to emotions described by human addicts during withdrawal. Moreover, it must be recalled that stressful situations cross-sensitize with effects of psychostimulants or opiates on behavioral sensitization. Chronic stress may therefore also induce an uncoupling between noradrenergic and serotonergic systems and thus be one source of mental illnesses such as bipolar disorder.

Materials and Methods

Animals. WT mice were C57BL6 male adults (25–35 g). Mice lacking the α1b-adrenergic receptor (α1b-AR KO) (12) or the $5HT_{2A}$ receptor ($5HT_{2A}$-R KO) (13) were backcrossed on a C57BL6 genetic background for at least seven generations. They were maintained on a 12-h light/dark cycle (lights on at 0700 hours) with food and water freely available.

Autoradiography. Brains were rapidly removed after animal death and frozen in isopentane (−40°C). Sections (20 μm) were cut with a cryostat, mounted onto gelatin-coated glass slides, and stored at −20°C until incubation. For α1b-AR binding sites, sections were incubated at 20°C with tritiated prazosin (1 nM) for 30 min in a 50 mM Tris·HCl (pH 7.4) buffer, washed five times in ice-cold buffer, and dried. For $5HT_{2A}$-R binding sites, sections were incubated at 20°C with tritiated ketanserin (2 nM) for 60 min in a 170 mM Tris·HCl, 10 mM pargyline, 4 mM $CaCl_2$, and 0.01% ascorbic acid (pH 7.4) buffer, washed five times in ice-cold buffer, and dried. Specificity was tested by adding during incubation 1 μM prazosin for α1b-AR binding sites or 1 μM SR46349B for $5HT_{2A}$-R binding sites. All slides were exposed to tritiated Hyperfilm for 45 days. Autoradiograms were revealed, digitized, and quantified by using IMAGEJ software.

Drugs. *d*-Amphetamine sulfate and PCA hydrochloride (Sigma Aldrich) were dissolved in saline. Prazosin hydrochloride (Sigma Aldrich) was sonicated in water. SR46349B hemifumarate [(1Z,2E)-1-(2-fluoro-phenyl)-3-(4-hydroxyphenyl)-prop-2-en-one-*O*-(2-dimethylamino-ethyl)-oxime hemifumarate] was a generous gift from Sanofi-Synthelabo Research (Montpellier, France). It was dissolved with a drop of lactic acid, neutralized with 1 M NaOH, and sonicated in saline. All drugs were injected i.p. (0.3 ml per 100 g). Doses are expressed as salts. *d*-Amphetamine was given at 2 mg/kg, and PCA was given at 7 mg/kg (17). Doses of prazosin (1 mg/kg i.p.) and SR46349B (1 mg/kg i.p.) were kept identical to previous experiments (11).

Locomotor Activity. ***Acute treatment.*** Mice were introduced in a circular corridor (4.5-cm width, 17-cm external diameter) crossed by four infrared beams (1.5 cm above the base) placed at every 90° (Imetronic, Pessac, France). The locomotor activity was counted when animals interrupted two successive beams and thus had traveled a quarter of the circular corridor. Spontaneous activity was

recorded for 60 min (habituation to the experimental procedure), and then mice were injected i.p. with *d*-amphetamine or PCA, and locomotor responses were recorded for an additional 200-min period.

Repeated treatment. Mice were injected on 4 consecutive days with *d*-amphetamine, and their locomotor activity was recorded after a *d*-amphetamine injection after a 4-day withdrawal with the same protocol as for an acute treatment. To test the effect of the pretreatment on the development of behavioral sensitization, each day mice received a pretreatment (saline, prazosin, SR46349B, or prazosin plus SR46349B) 30 min before the injection of *d*-amphetamine. When animals received prazosin as a pretreatment, two subsequent injections of prazosin were administered at 60 and 150 min after *d*-amphetamine injection because of the short half-life (100 min) of prazosin (11). Finally, locomotor responses to *d*-amphetamine were tested either 4 days or 1 month after the last *d*-amphetamine injection. Control mice were injected on 4 consecutive days with 0.9% saline (0.1 ml per injection), and their locomotor response to *d*-amphetamine (or PCA) was recorded 4 days later.

Surgery. Mice were anesthetized with sodium pentobarbital (60 mg/kg; Sanofi Santé Animale, Libourne, France) and placed in a stereotaxic frame (Kopf Instruments). Unilateral permanent cannula (CMA/7 guide cannula; Microdialysis, Solna, Sweden) was placed at the edge of the PFC and was secured on the skull with screw and dental cement. The coordinates for the guide cannula tip were as follows: anteroposterior, +2.6 relative to bregma; mediolateral, +0.5; dorsoventral, 0 mm from dura (31).

After surgery, mice were placed in individual plastic cages and allowed to recover for at least 4 days.

Microdialysis Experiment. ***Acute treatment.*** The day of the experiment, the microdialysis probe was inserted in the PFC (CMA/7; membrane length, 2 mm; diameter, 0.24 mm; cutoff, 6,000 Da; Microdialysis). Artificial CSF (147 mM NaCl/3.5 mM KCl/1 mM $CaCl_2$/1.2 mM $MgCl_2$/1 mM NaH_2PO_4/25 mM $NaHCO_3$, pH 7.6) was perfused with a CMA100 microinjection pump through the probe at a rate of 1 μl/min via fluorinated ethylene propylene (FEP) catheter (internal diameter, 0.12 mm) connected to a fluid swivel. Adequate steady state of monoamines levels in perfusate samples was reached 140 min after probe insertion, and samples were collected in 300-μl vials placed in a refrigerated, computer-controlled fraction collector (CMA/170). Samples (20 μl every 20 min) were collected during 100 min to determine basal extracellular monoamine values. After *d*-amphetamine or PCA injection, samples were collected for 200 min. Samples were analyzed the day of the experiment.

Repeated treatment. Mice received four consecutive daily injections of pretreatment plus *d*-amphetamine and waited 4 days or 1 month before the dialysis experiment. Every day after *d*-amphetamine injection, mice were immediately placed for 2 h in the cylindrical compartment used to perform microdialysis. Control mice received four consecutive daily injections of 0.9% saline (0.1 ml per injection) and waited 4 days before the dialysis experiment.

Biochemistry. Dialysate samples were completed to 30 μl with the adapted mobile phase and placed into a refrigerated automatic injector (Triathlon; Spark Holland, Emmen, The Netherlands). Twenty-five microliters of the sample were injected every 30 min through a rheodyne valve in the mobile phase circuit. High-performance liquid chromatography was performed with a reverse-phase column (80 × 4.6 mm, 3 μm particle size, HR-80, ESA, Chelmsford, MA). Mobile phase (for NE analysis: 0.1 M NaH_2PO_4/0.1 mM EDTA/3.8 mM octane sulfonic acid/0.25 mM triethylamine/10% methanol, pH 2.9; for 5-HT analysis: 0.1 M NaH_2PO_4/0.1 mM EDTA/1.5 mM octane sulfonic acid/0.25 mM triethylamine/15% methanol/5% acetonitrile, pH 2.9) was delivered at 0.7 ml/min by an ESA-580 pump (ESA). Electrochemical detection was performed with an ESA coulometric detector (Coulochem II 5100A, with a 5014B analytical cell; Eurosep, Cergy, France). The conditioning electrode was set at −0.175 mV, and the detecting electrode was set at +0.175 mV, allowing a good signal-to-noise ratio. External standards were regularly injected to determine the stability of the sensitivity (0.2 pg for NE and 0.3 pg for 5-HT).

Histology. At the end of the experiment, brains were put into a formaldehyde solution and cut on a microtome in serial coronal slices according to the atlas of Paxinos and Franklin (31). Histological examination of cannula tip placement was subsequently made on 100-μm safranine-stained coronal sections.

Statistics. Statistical analysis was performed by using PRISM 3.0 software (GraphPad, San Diego). Data from microdialysis and locomotor activity experiments were described as a function of time. Data from microdialysis were expressed as a percentage of the respective mean basal value. The extracellular monoamines level and the locomotor activity obtained after the *d*-amphetamine or PCA injection (100-min analysis) were compared and analyzed with two-way ANOVA (repeated measures). Pharmacological treatments correspond to independent groups of animals. Optical density data of the different mice were compared by using a two-tailed unpaired Student *t* test. Significant differences were set at $P < 0.05$.

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